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JAK3 and STAT3 genetic alteration as a treatment target in extranodal NK/T cell lymphoma, nasal type (NTCL)

NK/T-세포 림프종에서, 치료 표적으로서 JAK3 및 STAT3 유전변화 연구

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JAK3 and STAT3 genetic alteration as a treatment target in extranodal NK/T cell lymphoma, nasal type (NTCL)

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ABSTRACT

Introduction: Inhibition of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has been implicated as a treatment option for extranodal NK/T-cell lymphoma, nasal type (NTCL). However, JAK-STAT pathway alterations in NTCL are variable, and the efficacy of JAK-STAT pathway inhibition has been poorly evaluated.

Materials and Methods: JAK3 mutation and STAT3 genetic alterations were investigated by direct sequencing and immunohistochemistry in 84 patients with newly diagnosed NTCL. Novel JAK3 mutations were functionally validated using Ba/F3 and NIH-3T3 cells with retrovirus vector systems. A JAK3 homology model was constructed. Cell viability assays were performed using JAK3 or STAT3 inhibitor in NTCL cells.

Results: Five (7.0%) of 71 NTCL patients had *JAK3* mutations in the pseudokinase domain: 2 $JAK3^{A573V}$, 2 $JAK3^{H583Y}$, and 1 $JAK3^{G589D}$ mutation. Ba/F3 cells transduced with novel *JAK3* mutations ($JAK3^{H583Y}$ and $JAK3^{G589D}$) grew independently without IL-3. NIH-3T3 cells transduced novel *JAK3* mutations showed anchorage independent growth in soft agar plate. The transduced Ba/F3 cells were inhibited by the JAK3 inhibitor tofacitinib (mean IC_{50}, 85 ± 10nM and 54 ± 9nM). Ribbon diagrams showed that these *JAK3* pseudokinase domain mutations were located at the pseudokinase-kinase domain interface. Although phosphorylated STAT3 was overexpressed in 35 (51.4%) of 68 NTCL patients, *STAT3* mutation (p.Tyr640Phe; $STAT3^{Y640F}$) at the SRC homology 2 domain was detected in 1 (1.5%) of 63 patients. A STAT3 inhibitor was active against *STAT3*-mutant SNK-6 and YT cells.

Conclusions: Novel JAK3-activating mutations are oncogenic and sensitive to a JAK3 inhibitor in NTCL. Although STAT3 mutation rate is low in NTCL patients, STAT3-mutant NTCL cells are sensitive to a STAT3 inhibitor. JAK3 or STAT3 signal was altered in NTCL and pathway inhibition might be a therapeutic option for patients with JAK3- or STAT3-mutant NTCL.

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Keywords: NK/T-cell lymphoma, JAK3 mutation, STAT3 mutation

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INTRODUCTION

NK/T-cell lymphoma, nasal type (NTCL) is a subtype of Epstein-Barr virus (EBV)-positive and highly aggressive mature NK/T-cell neoplasm with extranodal presentation.[1,2] The distribution of NTCL is geographically different, consisting of about 10% of all peripheral T cell lymphoma and about half of all mature T cell lymphoma in Asian countries.[3] It also has unique clinical features being frequently localized in nasal cavity with tissue infiltration. The main treatment is combination of cytotoxic chemotherapy with or without radiation. Although the recent advances of treatment, the survival outcome of advanced disease is poor.[4] Therefore, discovery of new druggable targets is critical.[5]

With the advances of understanding molecular mechanism, many genetic alterations have been discovered in cancer. However, few molecular mechanisms have been understood and identifying the key pathway for treatment target is a major challenge in NTCL. Recently, several reports shed light on the genetic mechanism that JAK-STAT pathway activation[6-8] and other several novel oncogenes[9,10] may be implicated in NK/T-cell lymphoma oncogenesis. JAK/STAT pathway is well known

in hematopoiesis process and immune regulation.[11,12]

The frequency of JAK3 mutations or STAT3 alteration in NTCL has been variable. Several studies reported that JAK3 mutation or constitutive phosphorylation of STAT3 contribute to NK/T-cell lymphoma oncogenesis from 0 up to 35.4% of patients.[7,13,14] However, other reports using next-generation sequencing revealed a low JAK3 mutation frequency (0-5%).[8-10]

A JAK3 inhibitor was shown to be effective against NK-S1 cells with the *JAK3*^{A572V} mutation[7] and against MEC04 cells with the *JAK3*^{A573V} mutation[13] *in vitro* and *in vivo*; however, its ability to inhibit other *JAK3* mutant subtypes has not been elucidated.

In this study, we explored JAK3 mutations and STAT3 alterations in NTCL and discovered novel JAK3-activating mutations. We further investigated if the novel mutations had oncogenic potential, and the JAK3 mutations or STAT3 alteration were druggable by the JAK3 or STAT3 inhibitor

Materials and Methods

Patients

Eighty-four patients were newly diagnosed with NTCL based on the World Health Organization (WHO) classifications[1,2] between January 2001 and December 2011 at Seoul National University Hospital. Formalin-fixed, paraffin-embedded (FFPE) tissue samples were prepared and tested for JAK-STAT pathway alterations. This study was approved by the Institutional Review Board of Seoul National University Hospital (H1404-071-572).

JAK3 and *STAT3* genetic alterations

Direct sequencing of PCR products was performed bidirectionally to detect *JAK3* mutations on exons 13 and 16 and *STAT3* mutation on exon 21. Briefly, genomic DNA was isolated from FFPE tumor tissue using a Maxwell 16 FFPE Plus Tissue LEV DNA Purification kit (Promega, Madison, WI, USA). *JAK3* and *STAT3* exons were amplified from genomic DNA using EconoTaq PLUS GREEN 2× premix (Lucigen, Middleton, WI, USA), and then sequenced bi-directionally using

the ABI3730 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). The primer pairs used in the present study were as follows: *JAK3* A572V and A573V mutations on exon 13 (F: 5′-GCAGGTCTGTGAGCACAAAAT-3′, R: 5′-ACTGTCTCC AGCCATGCAC ACG-3′), *JAK3* V722I mutation on exon 16 (F: 5′-AGGCGCAGACACTTAGCTTG-3′, R: 5′-CAA AGTGGGGGTTCGGAGAC-3′), and *STAT3* Y640F and D661Y mutations on exon 21 (F: 5′-TTTCCGAATGCCTCCTCCT TGG-3′, R: 5′-GAGA TGACCTAGCTGTAGGTT CC-3′).

Retrovirus packaging and transduction

Retroviral vectors harboring novel *JAK3* mutations were generated through site-directed mutagenesis, and were introduced into Ba/F3 and NIH-3T3 cells to test transformation capacity. The coding region of *JAK3* (NM_000215.3) was amplified and cloned into vector TOPO TA (Invitrogen, Grand Island, NY, USA). Mutations of *JAK3* with 1847 T > C and 1866 A > G in the coding region were created by site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA) with mutation-

specific primers according to the manufacturer`s instructions. The Platinum-E retroviral packaging cell line was seeded in DMEM with 10% (v/v) FBS. The following day, the cells were transfected with the *JAK3* mutant construct in the pMXs-Puro vector (Cellbio Labs, San Diego, CA, USA) and the packaging plasmids pCL-eco using Fugene6® transfection reagents (Roche, Indianapolis, IN, USA). Viral supernatants were collected 48-72h later. Ba/F3 and NIH-3T3 cells in 6-well plates or 10-cm dishes were inoculated with 1 ml viral supernatant in 1 ml medium with polybrene (4 μg/ml). The transduced cells were selected using puromycin.

Soft agar assay

Cells were suspended in medium containing 0.3% select agar and plated on a bottom layer of medium containing 1% select agar [2% agar was melted in a microwave oven, mixed 1:1 with medium (2× DMEM medium with 20% FBS, 2% P/S mixture, and 2% glutamine)] in a 6-well plate. To enable colony formation, plates were incubated at 37°C for 2 weeks. The number of colonies that grew in the 6-well plates after 2 weeks was counted in triplicate and normalized to NIH-3T3 cells.

Immunohistochemistry and immunoblotting

Two-millimeter diameter core samples were taken from representative FFPE tissue blocks of patients' tumors. Tumor cell content (%) was evaluated by board-certified pathologists (YKJ, SJN, and CWK) (Table 1). Immunohistochemical staining for phosphorylation of STAT3 (p-STAT3) was performed using anti-pSTAT3 antibody (Cell Signaling Technology, Danvers, MA, USA). Part of the unstained tumor area was used as a negative staining control. Immunoreactivity was recorded as a total percentage (%) of positive cells for pSTAT3. Only tumor cells were counted; if the number of cells with strong nuclear staining for p-STAT3 was more than 2% of all tumor cells, then the tumor had a high level of expression. Antibodies to total STAT3 and phospho-STAT3 (Cell Signaling Technology) were used for Western blotting, and the blots were subsequently washed, transferred to membranes, and developed using Enhanced Lumi Light Western Blotting Substrate (Roche, Indianapolis, IN).

Cell culture and cell viability assay

SNK-6 and NKL cells were kindly provided by Drs. Norio Shimizu (Tokyo Medical and Dental University, Japan) and Masao Seto (Aichi Cancer Center, Japan), respectively. YT cells were purchased from the German Resource Center for Biological Material (DSMZ; Braunschweig, Germany). The JAK3 inhibitor tofacitinib or the STAT3 inhibitor Stattic was added to each well at specified concentrations (tofacitinib and Stattic, Selleck Chemical, Houston, TX, USA). The plates were incubated at 37°C in a 5% CO_2 atmosphere incubator for 48 or 72 h. For Ba/F3 with tofacitinib, the cells were incubated without IL-3 for 72 h; for NTCL with Stattic, the cells were incubated without IL-2 for 48 h. Growth inhibition assays were assessed using the CCK-8 colorimetric assay (DOJINDO Laboratories, Japan). For IL-3-independent growth assays, Ba/F3 cells transfected with the *JAK3* mutant plasmid were cultured in the absence of IL-3 for 6 days. The number of viable cells was determined by trypan blue exclusion assay.

JAK3 homology modeling

A JAK3 homology model was constructed using the SWISS-MODEL server.[15] TYK2 structure (Protein Data Bank ID: 4OLI)[16] was used as the modeling template. The stereochemical quality of the constructed models was validated using a Ramachandran plot generated by PROCHECK. Ribbon diagrams were generated using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA).

Statistical analysis

Mann Whitney U test was performed to compare differences of colony formation in soft agar. The differences in clinical characteristics according to the PD-L1 expression were analyzed using Fisher′s exact test. Progression-free survival (PFS) was measured from the date of initiation of treatment to disease progression, death, or the last follow-up. Overall survival (OS) was measured from the date of diagnosis to death or the last follow-up. Survival was analyzed using Kaplan-Meier plots and was compared with the log-rank test. Cox proportional hazard regression

model was used to perform multivariate analysis. A *P* value less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed using SPSS statistics software (version 20) (IBM Corp., Chicago, IL, USA).

Results

Patient characteristics

Tumor tissues from 84 NTCL patients were examined for the presence of *JAK3* mutations within the pseudokinase domain (JH2) and *STAT3* alterations (mutation within the SRC homology 2 domain and phosphorylation). All patients were EBV positve and about two-fifths of patinets were Ann Arbor stage III or IV (n=32). Approximately two-thirds of NTCL patients presented with involvement of the upper aerodigestive tract (n = 58), and two-fifths of patients presented with advanced disease (n = 32). (Table 1).

Table 1. Clinicopathologic Characteristics of Patients

Characteristic	No. of patients available	No. (%)
Age (years)		
Median	84	49
Range		8–79
Presentation		
UAT	84	58 (69)
NUAT		26 (31)
B symptoms		
No	83	49 (59)
Yes		34 (41)
ECOG performance status		
0–1	82	60 (73)
≥2		22 (27)
EBV		
Positive	84	84 (100)
Negative		0 (0)
Local tumor invasiveness		
No	84	48 (57)
Yes		36 (43)
Ann Arbor stage		
I, II	82	50 (61)
III, IV		32 (39)
IPI scores		
0–1		44 (54)
2	82	8 (10)
3		15 (18)
4–5		15 (18)
JAK3 mutation in exon 13	71	
Negative		66 (93)
Positive		5 (7)
JAK3 mutation in exon 16	68	
Negative		68 (100)
Positive		0 (0)
STAT3 mutation		
Negative	63	62 (98)
Positive		1 (2)
STAT3 phosphorylation		
Low	68	33 (49)
High		35 (51)
Tumor cell content (%)		
Median	83	35
Range		1-64

Abbreviation : UAT, upper aerodigestive tract; NUAT, non-upper aerodigestive tract; ECOG, eastern cooperative oncology group; EBV, epstein-barr virus; IPI , international prognostic index.

JAK3 mutations

We identified three *JAK3* mutations on exon 13 in 5 (7.0%) of 71 patients who had amplified PCR products from FFPE tumor tissues. Tumor cell content ranged from 1% to 64%. The *JAK3*^{A573V} mutation was identified in two patients with non-upper aerodigestive tract NTCL (Table 2). The following two novel heterozygous missense mutations were identified in *JAK3* exon 13: histidine-to-tyrosine substitution at codon 583 (*JAK3*^{H583Y}, c.1847C>T, NM_000215.3) (n = 2) and glycine-to-aspartic acid substitution at codon 589 (*JAK3*^{G589D}, c.1866G>A) (n = 1) (Figure 1A). Germ line *JAK3* mutation status was not evaluable due to lac k of normal tissues or blood samples except patient #5. The sequencing of *JAK3* in bone marrow sample showed no germ line *JAK3* mutation (Figure 1B). Therefore, the *JAK3*^{G589D} mutation was somatic. The *JAK3* mutations in exon 16, such as *JAK3*^{V722I},

were not observed in 68 NTCL patients who had amplified PCR products from FFPE tumor tissues. Overall, the *JAK3* mutation frequency in NTCL was 7.0% in our study (Figure 2)[7-10,17,18]. Ki-67 level was not different between mutation positive and negative group (p=0.765)

Table 2. JAK3 or STAT3-mutant NTCL

No.	#1	#2	#3	#4	#5	#6
Sex/age	F/49	M/37	M/68	M/47	F/42	M/57
Primary site	NUAT	NUAT	NUAT	UAT	UAT	UAT
Stage	I	IV	III	IV	I	IV
First line treatment	CHOP	IMEP	CHOP	BVP	IMEP	IMEP
Response to treatment	SD	PD	NA	PD	CR	PD
PFS(months)	4.07	2.43	NA	0.6	29.27	2.03
OS(months)	11.67	2.83	NA	0.7	29.77	3.36
Ki-67(%)	68	20	30	70	60	70
JAK3 mutation	A573V	A573V	H583Y	H583Y	G589D	WT
STAT3 mutation	WT	NA	WT	NA	WT	Y640F
Phospho-STAT3	High	Low	Low	Low	Low	Low

Abbreviation : UAT, upper aerodigestive tract; NUAT, non-upper aerodigestive tract; NA, not available; WT, wild-type; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisolone; IMEP, ifosfamide, methotrexate, etoposide, and prednisolone; BVP, bleomycin, vincristine, prednisolone.

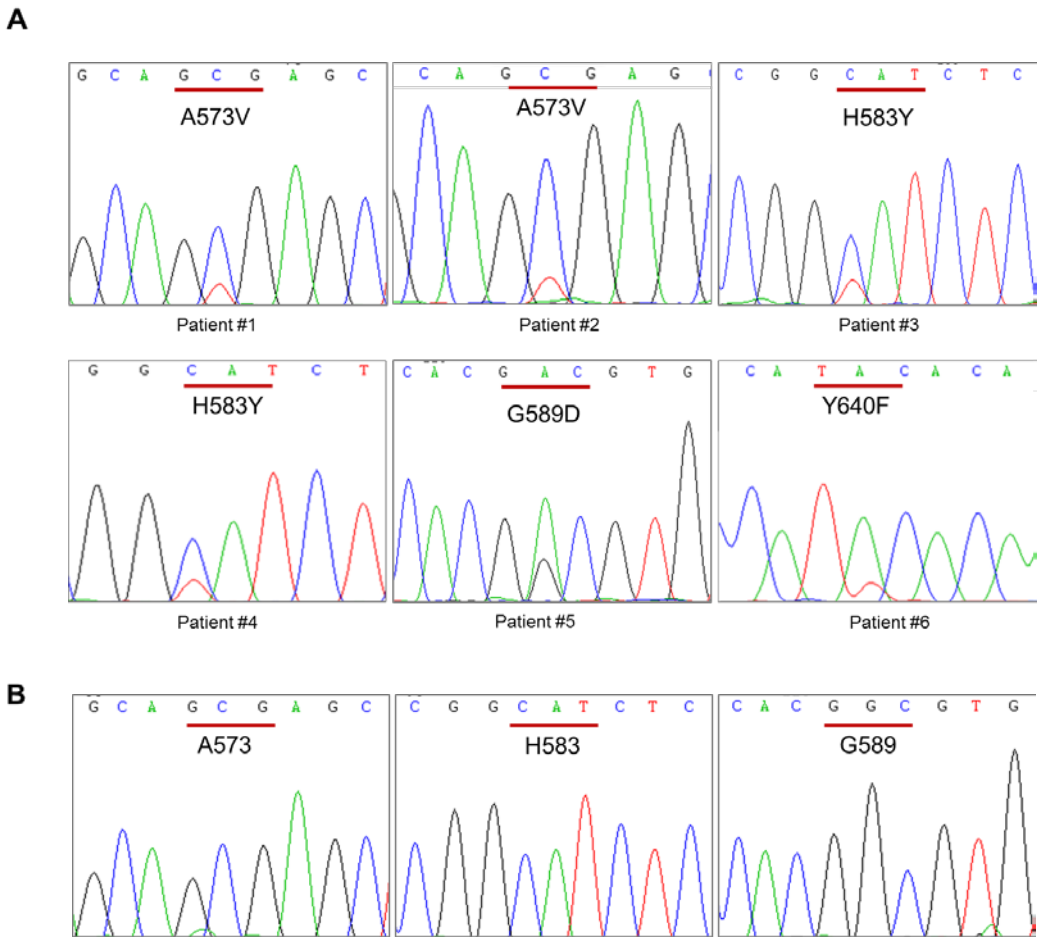


Figure 1. Electropherogram of JAK3 and STAT3 mutation. A. JAK3 mutation (patient #1-5) and STAT3 mutation (patient #6) in NTCL. B. Electropherogram of wild –type *JAK3* in a normal bone marrow sample of Patieht#5(with G589D mutation). Sanger sequencing of JAK3 genomic DNA from bone marrow sample showed that the no amino acid changed in 3 mutation site.

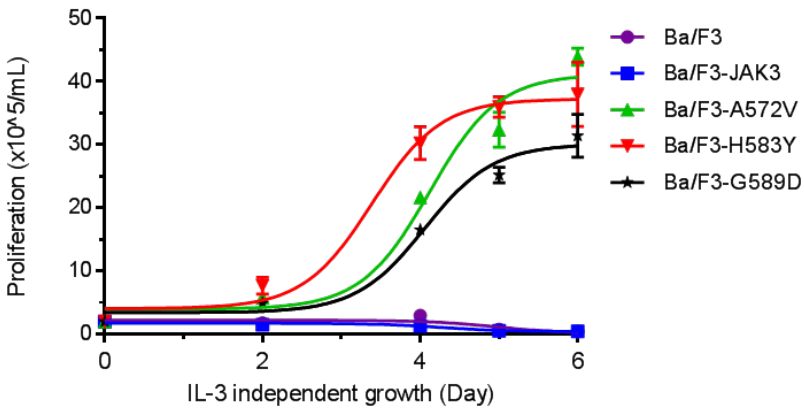
Study	**A572V**	**A573V**	**H583Y**	**G589D**	**V722I**	***JAK3* mutation (%)**
Koo et al.[7]	20/65	4/65	0/65	0/65	0/65	23/65 (35.4%)
Bouchekioua et al.[17]	0/19	3/19	0/19	0/19	1/19	4/19 (21%)
Kimura et al.[18]	0/17	0/17				0/17 (0%)
Lee et al.[10]	2/39	0/39				2/39 (5.1%)
Küçük et al.[8]	0/40	0/40	0/40	0/40	0/40	0/40 (0%)
Jiang et al.[9.]	0/25	0/25	0/25	0/25	0/25	0/25 (0%)
Present study	0/71	2/71	2/71	1/71	0/68	5/71 (7.0%)
Sum (%)	22 (8.0)	9 (3.1)	2 (0.9)	1 (0.4)	1 (0.4)	34/276 (12.3)

Figure 2. Frequency of *JAK3* mutation in published data and the location of *JAK3* mutation.

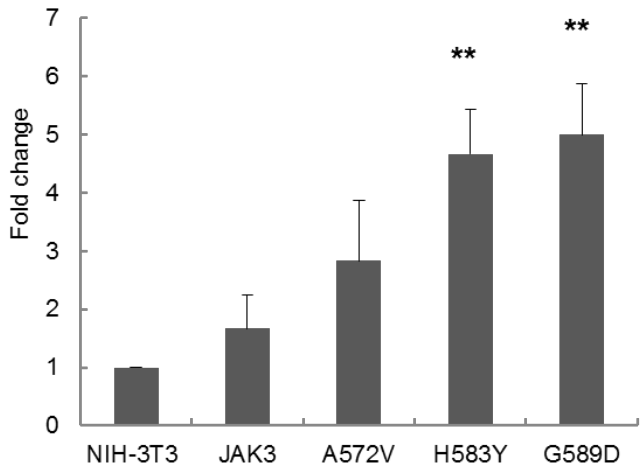
Oncogenic properties of novel *JAK3* mutations

We constructed retroviral vectors expressing *JAK3* wild type, or *JAK3*^{H583Y}, *JAK3*^{G589D}, or *JAK3*^{A572V} mutations. Then we inoculated NIH-3T3 or Ba/F3 cells with these vectors. Ba/F3-*JAK3*^{H583Y} and Ba/F3-*JAK3*^{G589D} cells displayed IL-3-independent growth (Figure 3A), whereas NIH-3T3-*JAK3*^{H583Y} and NIH-3T3-*JAK3*^{G589D} cells displayed anchorage-independent growth (Figure 3B), similar to the transforming activity of *JAK3*^{A572V} mutant cells.[7,10] Then, we tested whether the JAK3 inhibitor tofacitinib could inhibit the growth of *JAK3*^{H583Y}- and *JAK3*^{G589D}-transduced Ba/F3 cells. Tofacitinib significantly inhibited Ba/F3-*JAK3*^{H583Y} and Ba/F3-*JAK3*^{G589D} cell growth; it also inhibited Ba/F3-*JAK3*^{A572V} growth, but to a lesser extent (Figure 3C).

A


Proliferation (x10^5/mL)
50
40
30
20
10
0
0
2
4
6
IL-3 independent growth (Day)
Ba/F3
Ba/F3-JAK3
Ba/F3-A572V
Ba/F3-H583Y
Ba/F3-G589D

B


Fold change
7
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NIH-3T3
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H583Y
G589D

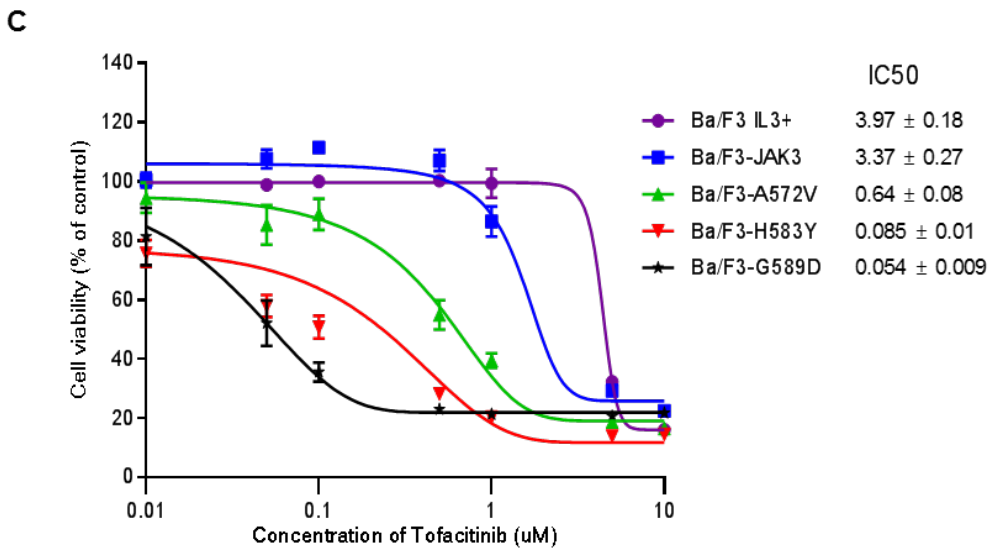


Figure 3. Oncogenic properties of novel *JAK3* mutations and Cell viability assays with tofacitinib. A: IL-3-independent proliferation of Ba/F3 cells transduced with retrovirus containing JAK3 mutation. B: NIH-3T3-JAK3, NIH-3T3-A572V, NIH-3T3-H583Y, and NIH-3T3-G589D cell growth was measured by colony formation in soft agar. C: Ba/F3-JAK3H583Y and Ba/F3-JAK3G589D cells were sensitive to the JAK3 inhibitor. IC50 values are provided for each case (μM).

**denotes $P < 0.05$, compared with NIH-3T3-JAK3.

Structural modeling of novel *JAK3* mutations

Ribbon diagrams of the *JAK3* JH2 and kinase (JH1) domains showed that H583 in the αC-helix and G589 in the β4-sheet were located in the N-lobe of the JH2 domain (Figure 4A), similar to other tumor-associated *JAK3* JH2 mutations, such as A572V and A573V. The two mutations are located in the interface between the pseudokinase and kinase domains. The *JAK3*^{H583Y} and *JAK3*^{G589D} mutation sites were closer to the JH1 domain than the *JAK3*^{A572V} and *JAK3*^{A573V} mutation sites (Figure 4B, 4C).

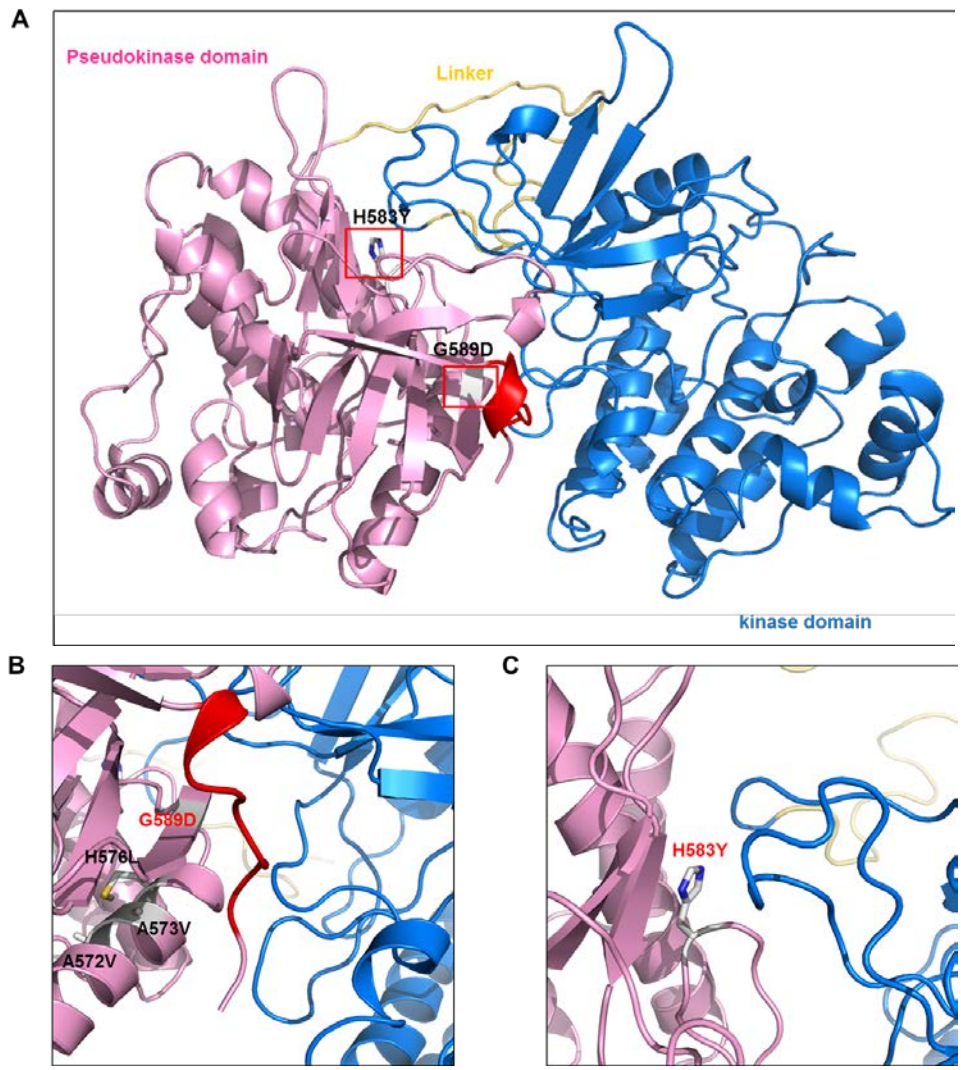


Figure 4. Homology models of JAK3 pseudokinase and kinase domain (A). H583Y (B) and G589D (C) are located in the interface between the two domains.

STAT3 mutation and phosphorylation

Strong expression of phosphorylated STAT3 (p-STAT3) in the nucleus of ≥2% of all tumor cells was observed in 35 (51.4%) of 68 patients (Figure 5). Although clinical variables were not significantly associated with p-STAT3 expression, tumors with high p-STAT3 expression showed a trend toward better ECOG performance status but toward advanced disease stage (Table 3). The Kaplan-Meier plot showed high pSTAT3 showed better survival but it was not statistically significant (P=0.137) (Figure 6). The univariate and multivariate analysis showed local invasion, ECOG performance status and IPI score was significantly associated with overall survival (Table 4, Table 5). However, p-STAT3 status did not affect overall survival.

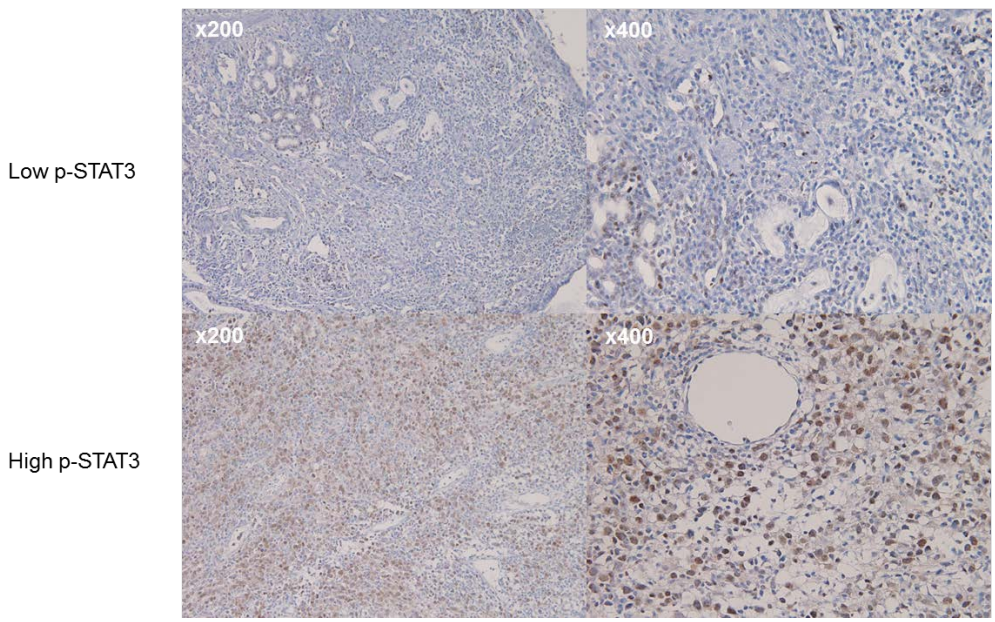


Figure 5. p-STAT3 in NTCL. Immunohistochemical staining for STAT3 phosphorylation (p-STAT3) reveals low p-STAT3 (upper panel) and high p-STAT3 (lower panel) expression.

Table 3. phospho-STAT3 and clinical characteristics

		pSTAT3 high	**pSTAT3 low**	**p**
Age mean		48.7	46.0	0.522
SEX	M F	20 15	21 12	0.584
Presentation	UAT NUAT	25 10	23 10	0.876
B Symptom	Yes No	15 20	14 19	0.971
ECOG	0-1 ≥2	29 6	20 11	0.089
Local Invasion	Yes No	14 21	15 18	0.649
Ann Arbor Stage	I, II III, IV	18 17	23 9	0.086
IPI scores	0,1,2 ≥3	23 12	22 10	0.792

Abbreviation : UAT, upper aerodigestive tract; NUAT, non-upper aerodigestive tract;

ECOG, eastern cooperative oncology group; IPI , international prognostic index

Table 4. Univariate analysis of survival

		Univariate analysis		
Variable		HR	95%CI	P-value
Age	<65 ≥65	0.784	0.301-2.042	0.618
sex	M F	0.766	0.370-1.584	0.472
Local invasion	Yes No	0.439	0.215-0.898	0.024
Ki-67(<45)	<45 ≥45	0.831	0.510-2.315	0.831
Ann Arbor Stage	0,1,2 ≥3	3.558	1.688-7.500	0.001
B Symptom	Yes No	0.270	0.129-0.564	0.001
Primary tumor location	UAT NUAT	2.131	1.033-4.397	0.041
ECOG(01 vs other)	0,1 ≥2	9.211	4.375-19.394	0.001
IPI (0,1,2 vs other)	0,1,2 ≥3	6.690	3.043-14.706	0.001
Mutation (JAK3 or STAT3)	Positive Negative	0.415	0.124-1.389	0.154
pSTAT	Positive Negative	1.792	0.831-3.860	0.137

Abbreviation : UAT, upper aerodigestive tract; NUAT, non-upper aerodigestive tract;

ECOG, eastern cooperative oncology group; IPI , international prognostic index

Table 5. Multivariate analysis of survival

Variable		**HR**	**95%CI**	**P-value**
Local invasion	Yes No	0.448	0.207-0.967	0.041
ECOG(01 vs other)	0,1 ≥2	4.332	1.915-9.801	0.001
IPI (1,2 vs other)	1,2 ≥3	4.538	1.869-11.018	0.001

Abbreviation : ECOG, eastern cooperative oncology group; IPI , international prognostic index

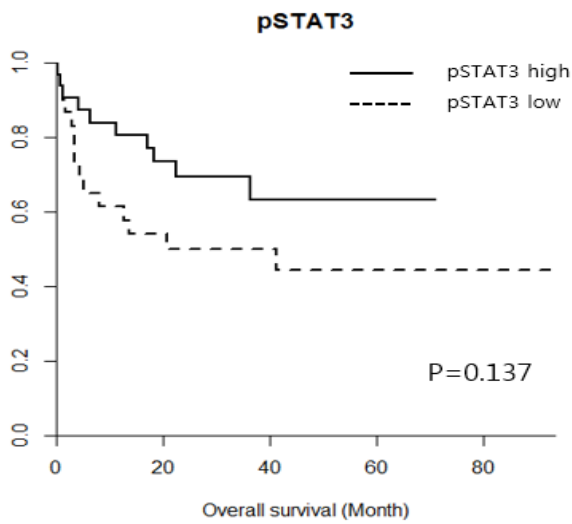
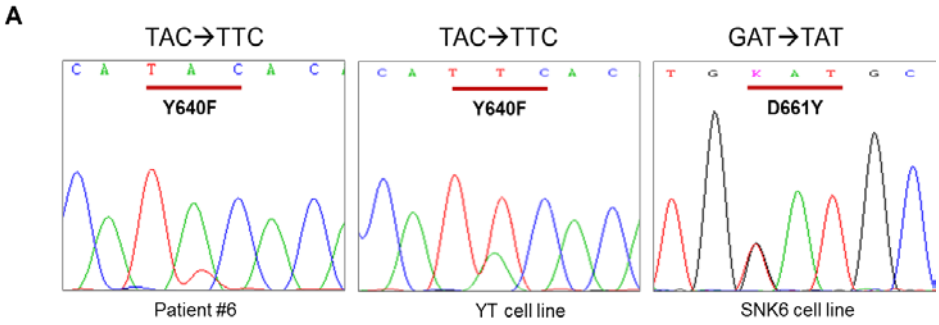
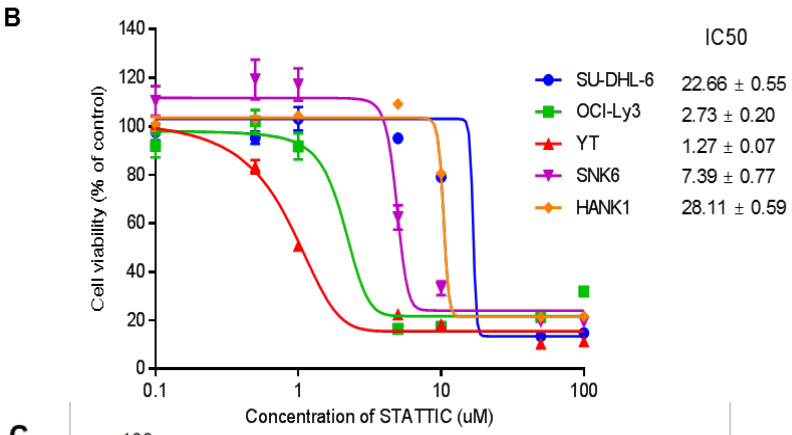
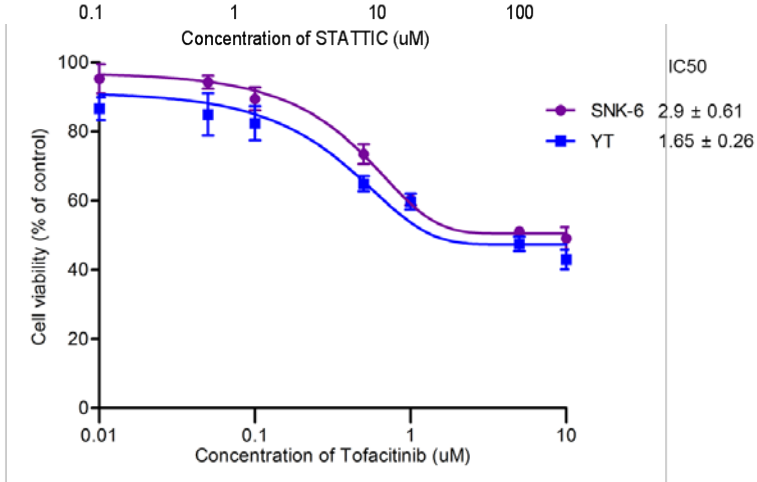


Figure 6. Kaplan-Meier plot according to pSTAT3. High pSTAT3 showed better survival but it was not statistically significant (p=0.137)

STAT3 mutation (p.Tyr640Phe; *STAT3*^{Y640F}) at the SRC homology 2 (SH2) domain was found in 1 (1.5%) of 63 tumor samples (Figure 7A). Growth of YT and SNK-6 cells harboring *STAT3*^{Y640F} and *STAT3*^{D661Y} mutations, respectively, were inhibited by the STAT3 inhibitor Stattic (Figure 7B), but not by tofacitinib (Figure 7C). Stattic was not toxic to HANK1 cells with wild-type STAT3, which have relatively lower p-STAT3 (Tyr705) levels than SNK-6 and YT cells (Figure 7D). Diffuse large B-cell lymphoma cells without *STAT3* mutation were used as controls; OCI-Ly3 cells with high p-STAT3 (Tyr705) expression were more sensitive to Stattic than SU-DHL6 cells with low p-STAT3 (Tyr705) expression (Figures 7B and 7D).


A
TAC→TTC
TAC→TTC
GAT→TAT
Y640F
Y640F
D661Y
Patient #6
YT cell line
SNK6 cell line


B
IC50
SU-DHL-6 22.66 ± 0.55
OCI-Ly3 2.73 ± 0.20
YT 1.27 ± 0.07
SNK6 7.39 ± 0.77
HANK1 28.11 ± 0.59
Cell viability (% of control)
Concentration of STATTIC (uM)


C
IC50
SNK-6 2.9 ± 0.61
YT 1.65 ± 0.26
Cell viability (% of control)
Concentration of Tofacitinib (uM)

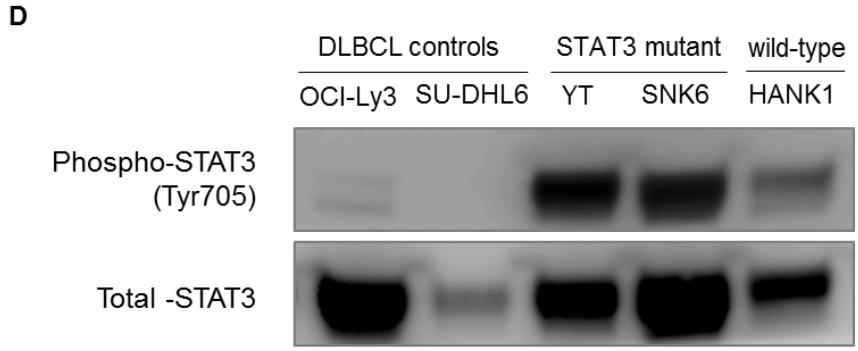


Figure 7. *STAT3* alteration and inhibition. **A:** Electropherogram of *STAT3* mutation in patient #6, YT cells and SNK6 cells. **B:** Cell viability assay with various Stattic concentrations in NTCL cells. YT and SNK6 harboring *STAT3* mutation were more sensitive to the STAT3 inhibitor than HANK1 or SU-DHL-6. Data are the mean ± standard deviation of three independent experiments. **C**: Cell viability assay with various tofacitinib concentrations in *STAT3*-mutant SNK-6 and YT cell lines. These were not sensitive to the tofacitinib. IC_{50} values were provided as mean ± standard deviation (μM). **D:** STAT3 phosphorylation status in various cell lines. OCI-Ly3 and SU-DHL6 derived from diffuse large B cell lymphoma were used as control cells.

Discussion

Our study demonstrated that *JAK3*-activating mutations were rare (7.0%) in patients with NTCL. We identified novel *JAK3*^{H583Y}and *JAK3*^{G589D} mutations that were oncogenic and targetable alterations. In addition, the *STAT3* mutation in the SH2 domain also might be a promising target for inhibition as an NTCL treatment.

Since the first report of an oncogenic *JAK3* mutation,[7] reports of its mutation frequency in NTCL has been variable. In contrast to the studies reporting high mutation frequencies (21-35%),[7,13] subsequent studies suggested a much lower mutation frequency ranging from 0% to 5.1%,[8,10,18] which was comparable to our result (Figure 2). Although the *JAK3*^{A573V} mutation (which is the second-most common) was identified in our patients, the *JAK3*^{A572V} and *JAK3*^{V722I} mutations were not observed, in contrast to previous reports.[7,13] This difference in *JAK3* mutation frequency might be attributable to the ethnic variation in NTCL. It is known that NTCL is heterogeneous and the incidence is variable according to geographic region.[3,4] This suggests ethnic difference could be implicated in the different

mutation frequency. In addition, technical issues in detecting mutations and variation in tumor content might have affected the results. Many cases of NK/T cell lymphoma have a high content of inflammatory cells, which might contribute to reported differences in mutation frequency.

In this study, novel *JAK3*^{H583Y} and *JAK3*^{G589D} mutations with oncogenic potential that were somatic and not seen in COMSIC and public databases, were identified in three patients. Anchorage- or IL-3-independent growth of *JAK3*^{H583Y} and *JAK3*^{G589D}-mutant cells was observed in the NIH-3T3 or Ba/F3 cell lines, which suggested these novel *JAK3* mutations had oncogenic properties. *JAK3* mutant cells exhibited 6.2-46-fold greater sensitivity to tofacitinib compared to *JAK3* wild-type cells. Considering the efficacy of tofacitinib for inhibiting JAK3 in Ba/F3-*JAK3*^{H583Y} and *JAK3*^{G589D} cells and inhibiting JAK3 through prevention of EZH2 phosphorylation in NTCL,[19] JAK3 inhibition might be clinically relevant for treating *JAK3*-mutant NTCL. In addition, structural modeling found that the novel *JAK3* mutations were located in the *JAK3* pseudo-kinase domain. The novel *JAK3* mutations had closer proximity to the JH1 domain compared to the *JAK3*^{A572V} and

JAK3^{A573V} mutations, which might render the novel *JAK3* mutations more oncogenic and sensitive to tofacitinib. These results suggested that novel *JAK3* mutations in the N-lobe of the JH2 domain are druggable target oncogenes located at the interface of the JH2 and JH1 domains, similar to other cancer-associated *JAK3* mutations.[16] Considering JH2-mediated negative regulation of JAK3,[20] our pathogenic JAK3 mutations in the JH2 domain might disrupt a negative signal between JH2-JH1 domains, resulting in JAK3 kinase activation and sensitivity to inhibition.[7] Tofacitinib is a potent inhibitor of JAK1 and JAK3, but not JAK2, and has been used as monotherapy for treatment of rheumatoid arthritis.[21] In addition, tofacitinib has shown clinical activities against *JAK3*-mutant primary mediastinal B-cell lymphoma as combination therapy[22] and *STAT3*-mutant T-cell large granular lymphocytic leukemia as monotherapy.[23] Clinical and *in vitro* activities of JAK3 inhibition warrant further investigation in *JAK3*-mutant lymphoid neoplasms including NTCL.

STAT3 genetic alterations were evaluated because of the oncogenic role of constitutive STAT3 signal activation in NTCL.[6,11] Although more than half of the patients showed strong p-STAT3 expression, the previously identified *STAT3*^{Y640F}

mutation[8-10,24] was found in only one patient, which was a relatively lower frequency than in other published studies (*STAT3* mutation frequencies in the SH2 domain were reported as 5.9%,[8] 7.6%,[9] and 26% [10]). The several factors affecting STAT3 phosphorylation and activation has been suggested, however, the exact mechanism of STAT3 phosphorylation in NTCL is not clear. Several studies reported that EBV associated protein such as LMP1 or EBNA2 could affect STAT3 activation in lymphoma.[25,26] Other studies showed LEP fusion gene or loss of SH2B33 was associated with increase of p-STAT3.[27] EZH2 phosphorylation was also associated with p-STAT3 in glioblastoma.[28] STAT3 activation was adversely associated with survival in diffuse large B-cell lymphoma patients,[29] whereas p-STAT3 did not correlate with survival in NTCL patients. Stattic, a STAT3 inhibitor, was effective against *STAT3*-mutant NTCL cells, but less effective in *STAT3*-wild type NTCL cells with high p-STAT3 expression in our study. Similarly, NTCL patient-derived MEC04 cells with high p-STAT3 expression were inhibited by high concentrations of a STAT3 inhibitor.[6] Interestingly, JAK1/2 inhibitors that disrupted the STAT3

activating signal inhibited NTCL cells with high p-STAT3 expression[6] as well as *STAT3*-mutant NTCL cells,[8] which suggested a therapeutic option for NTCL patients.

In conclusion, the frequency of *JAK3* mutations in the JH2 domain was relatively low in NTCL (mean, 11.8%; range 0-35.4%) in contrast to a previous report.[7] Here, we identified novel *JAK3*^{H583Y} and *JAK3*^{G589D}-activating mutations that were oncogenic and sensitive to a JAK3 inhibitor. Although the frequency of *STAT3* mutations was low in NTCL patients, *STAT3*-mutant NTCL cells were sensitive to a STAT3 inhibitor, but not to a JAK inhibitor. These results indicated that *JAK3* and *STAT3* mutations are candidate therapeutic targets in NTCL patients. Future efforts should be directed to evaluate the efficacy of JAK3 or STAT3 inhibitors in NTCL patients with *JAK3*- or *STAT3*-activating mutations.

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국문 초록

서론: Janus kinase (JAK)- Signal trasnsducer and activator of transcription(STAT) 경로를 억제하는 것은 NK/T세포 림프종 치료법 중 선택의 한가지로 고려되고 있다. 그러나, JAK-STAT 경로의 변화는 다양하고, 해당 경로의 억제 효능은 제대로 평가되어 있지 않다. 본 연구는 NK/T 세포 림프종에서, JAK3 유전자 돌연변이 및 STAT3 의 변화를 탐색하고, 새로이 발견된 JAK3 유전자 돌연변이의 종양형성 가능성을 평가하였다. 또한 JAK3 돌연변이 및 STAT3 의 변화가 치료약제의 표적이 될 수 있는지를 평가하였다.

방법 및 대상: NK/T 세포 림프종으로 새롭게 진단된, 84명 환자의 종양 조직에서 직접적인 시퀀싱 및 면역조직 화학염색을 시행하여, JAK3 유전자의 돌연변이 및 STAT3 유전자및 발현의 변화를 조사하였다. 새로운 JAK3 유전자 돌연변이는, 레트로바이러스 벡터 시스템을 사용한 Ba/F3 및 NIH-3T3세포주를 이용하여, 기능적 증명을 시행하였다. 또한 JAK3 상동 구조 모델을 제작하여, 유전자의 구조적 위치를 파악하였

다. NK/T 세포 림프종 세포주에서, JAK3 및 STAT3 억제제를 이용한 세포 생존능력 분석을 시행하였다.

결과: 71명의 NK/T 세포 림프종 환자 중, 5명(7.0%)에서, JAK3 유전자의 pseudokinase 도메인에 돌연변이 ($JAK3^{A573V}$ 2명, $JAK3^{H583Y}$ 2명, $JAK3^{G589D}$ 1명)가 발견되었다. 새로운 JAK3 돌연변이($JAK3^{H583Y}$ 및 $JAK3^{G589D}$)가 형질도입된 Ba/F3 세포주는 IL-3 와는 무관한, 독립적인 증식이 관찰되었다. 같은 돌연변이 유전자가 형질도입된 NIN-3T3 세포주는 soft agar plate 에서 비부착증식이 일어남을 확인하였다. 새로운 돌연변이 유전자들이 형질도입 Ba/F3 세포주는 JAK3 억제제인 tofacitinib을 처리하였을 경우, 세포 증식이 억제되었다(mean IC_{50}, 85 ± 10nM and 54 ± 9nM). 단백질 구조를 보여주는 리본 다이아그램(Ribbon diagram)에서, JAK3 pseudokinase 도메인에 위치한 JAK3 돌연변이들은 pseudokinase-kinase 접촉면에 위치하는 것이 확인되었다. 인산화 STAT3는 68명의 NK/T세포 림프종 환자중 35명(51.4%)에서 과발현되어 있었으나, STAT3 유전자 돌연변이(p.Tyr640Phe; $STAT3^{Y640F}$)는 63명 중 1명(1.5%)에서 관찰되었다. STAT3 억제제는 STAT3돌연변

이가 존재하는 SNK-6 및 YT 세포주에서 활성이 있었다.

결론: NK/T 세포 림프종에서, 새롭게 발견된 JAK3 활성 돌연변이는 종양형성 능력을 가지고, JAK3 억제제에 효과적으로 반응한다. STAT3 돌연변이 비율은 NK/T 세포 림프종에서 낮으나, STAT3-돌연변이 NK/T 림프종 세포주는 STAT3 억제제에 효과적으로 증식이 억제되었다.. JAK3 및 STAT3 신호 경로는 NK/T 세로 림프종에서 변형이 있으며, 해당 경로 억제제는 JAK3 및 STAT3 돌연변이가 있는 환자에서 선택 가능한 치료방법이 될 수 있다.

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